In vitro isolation from *Amblyomma ovale* (Acari: Ixodidae) and ecological aspects of the Atlantic rainforest *Rickettsia*, the causative agent of a novel spotted fever rickettsiosis in Brazil

M. P. J. SZABÓ[1,2]*, F. A. NIERI-BASTOS[1], M. G. SPOLIDORIO[1], T. F. MARTINS[1], A. M. BARBIERI[1] *and* M. B. LABRUNA[1]

[1] *Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Orlando Marques de Paiva, 87 Cidade Universitária, CEP: 05508-270, São Paulo, SP Brasil*
[2] *Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Uberlândia Av. Pará, 1720/Campus Umuarama-Bloco 2T, Uberlândia, Minas Gerais, Brasil*

(*Received 3 July 2012; revised 3 September, 13 October and 2 November 2012; accepted 5 November 2012; first published online 30 January 2013*)

SUMMARY

Recently, a novel human rickettsiosis, namely Atlantic rainforest spotted fever, was described in Brazil. We herein report results of a survey led around the index case in an Atlantic rainforest reserve in Peruibe municipality, southeastern Brazil. A *Rickettsia parkeri*-like agent (*Rickettsia* sp. Atlantic rainforest genotype) and *Ricketsia bellii* were isolated from adult *Amblyomma ovale* ticks collected from dogs. Molecular evidence of infection with strain Atlantic rainforest was obtained for 30 (12·9%) of 232 *A. ovale* adult ticks collected from dogs. As many as 88·6% of the 35 examined dogs had anti-*Rickettsia* antibodies, with endpoint titres at their highest to *R. parkeri*. High correlation among antibody titres in dogs, *A. ovale* infestations, and access to rainforest was observed. *Amblyomma ovale* subadults were found predominantly on a rodent species (*Euryoryzomys russatus*). From 17 *E. russatus* tested, 6 (35·3%) displayed anti-*Rickettsia* antibodies, with endpoint titres highest to *R. parkeri*. It is concluded that Atlantic rainforest genotype circulates in this Atlantic rainforest area at relatively high levels. Dogs get infected when bitten by *A. ovale* ticks in the forest, and carry infected ticks to households. The role of *E. russatus* as an amplifier host of *Rickettsia* to *A. ovale* ticks deserves investigation.

Key words: Atlantic rainforest spotted fever, *Amblyomma ovale*, Brazil, isolation, ecology.

INTRODUCTION

South America has witnessed a great expansion in rickettsiology and at least 6 *Rickettsia* species have been described in this continent for the first time since 2001 (Labruna, 2009; Pacheco *et al*. 2011). Among these species *Rickettsia parkeri* has gained much attention. This bacterium, isolated initially by R. R. Parker from *Amblyomma maculatum* ticks in the USA (Parker, 1939), was found to be pathogenic to humans more than 60 years later (Paddock *et al*. 2004). Human disease caused by this *Rickettsia* was shown to be milder than the highly fatal *Rickettsia rickettsii*-caused Rocky Mountain spotted fever. It was also shown to be clinically distinct by the presence of a cutaneous eschar at the tick-bite site as well as regional lymph node enlargement (Paddock *et al*. 2004; Cragun *et al*. 2010). *R. parkeri* was recently found and/or isolated from *Amblyomma triste* ticks in Uruguay, Brazil and Argentina (Venzal *et al*. 2004; Silveira *et al*. 2007; Nava *et al*. 2008). Actually, rickettsiosis due to *R. parkeri* has been recognized for almost 20 years in Uruguay but the infectious agent was misdiagnosed with *Rickettsia conorii* (Venzal *et al*. 2004; Conti-Diaz *et al*. 2009). Recently, human rickettsiosis caused by *R. parkeri* infection was also recognized in Argentina (Romer *et al*. 2011).

Important aspects link human *R. parkeri* infections in the USA, Uruguay and Argentina; they are transmitted by either *A. maculatum* or *A. triste*, tick species that maybe confounded by their morphological similarities (Mertins *et al*. 2010). Since *A. triste* is a species associated to flooded/marsh areas, human disease has distinct epidemiological features. Curiously in Brazil, in spite of the presence of *R. parkeri*-infected *A. triste* ticks (Silveira *et al*. 2007), not a single human disease due to *A. triste* bite has been reported so far. Nonetheless, recent observations have shown that a *Rickettsia* genetically related to *R. parkeri* is circulating among other tick species, in different environmental context and is causing human disease clinically related to the disease caused by *R. parkeri*. Spolidorio *et al*. (2010) reported a novel spotted fever group (SFG) agent of human rickettsioses in São Paulo State, southeastern Brazil, in an Atlantic rainforest reserve. Hence the disease was named Atlantic rainforest spotted fever, caused

* Corresponding author: Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Uberlândia Av. Pará, 1720/Campus Umuarama-Bloco 2T, Uberlândia, Minas Gerais, Brasil. Tel: +55 34 3218 2691. Fax: +55 34 3218 2521. E-mail: szabo@famev.ufu.br

doi:10.1017/S0031182012002065

by a *Rickettsia* genotype designated as Atlantic rainforest. Until this report, *R. rickettsi* was the only tick-borne *Rickettsia* species known to cause human disease in the country. DNA sequences of the Atlantic rainforest *Rickettsia* were amplified from the tick-bite lesion (eschar) and were shown to be a novel SFG strain closely related to *R. parkeri*, *Rickettsia africae* and *Rickettsia sibirica*. Even though the tick of this human case was not recovered, Sabatini *et al*. (2010) detected high levels of *Rickettsia* genotype in *Amblyomma ovale* ticks of dogs and vegetation in another Atlantic rainforest in the state of São Paulo. Subsequently, the same *Rickettsia* genotype was identified in the state of Bahia (northeastern Brazil) from a patient with febrile illness which, among others, included eschar at a tick-bite location, a regionally enlarged lymph node, and a rash (Silva *et al*. 2011). Furthermore *A. ovale, Amblyomma aureolatum* and *Rhipicephalus sanguineus* ticks were shown to be infected with the Atlantic rainforest genotype on the southern coast of Brazil, where dozens of clinical cases of spotted fever rickettsiosis have been reported in humans, although without determination of the *Rickettsia* species (Medeiros *et al*. 2011).

Herein we report the isolation, molecular characterization and ecological features of the Atlantic rainforest genotype from the location of the human index case in southeastern Brazil (Spolidorio *et al*. 2010).

MATERIALS AND METHODS

Study site

A survey was held in the area of the strain Atlantic rainforest index case, an Atlantic rainforest reserve (Estação Ecológica de Juréia-Itatins) in Peruibe municipality, São Paulo State, southeastern Brazil. This reserve is located by the seaside, approximately 200 km south of São Paulo city. Part of the reserve still has scattered houses embedded in the forest, and the index case occurred in one of them. The survey was led around this house (24°25′23·6″S; 47°03′06·6″W; elevation 23 metres above sea level). Samples were collected on 6 field campaigns (April, June, August, November, December 2010 and February 2011) over a 1-year period. The present study was previously approved by São Paulo State Forest Institute (letters 858/2010 and D86/2010 W), and by the Brazilian Environment Institute – IBAMA (Permit No. 22212-2).

Host-seeking ticks

Host-seeking ticks were collected from vegetation at a range of 10 km by either cloth dragging or visual inspection as described elsewhere (Terassini *et al*. 2010). For this purpose 2 trails and their ramifications in the forest and within the property of the index case house were sampled. Additionally, the vegetation on both sides of a small 4 km-long road (Estrada do Juquiazinho) and 2 trails in the forest by a beach (Praia do Juquiazinho) were also inspected at each campaign.

Small mammals

In each campaign small mammals were captured within a radius of 500 metres from the index case house with the aid of 75 live-traps (60 Sherman and 15 Tomahawk) during 4 consecutive nights. Trapped animals were then anaesthetized with ketamine and carefully examined for ticks. Tick-infested rodents were euthanized by deepening anaesthesia, and the spleen and lungs were collected and frozen in liquid nitrogen. Blood sample was collected from all animals by venipuncture. Carcasses of euthanized animals were sent to Zoology Museum of the University of São Paulo for identification and deposit of voucher specimens.

Dogs

All ticks from the dogs of 14 properties scattered within the reserve were collected on each campaign. It was observed whether animals were restricted to the properties by either fences or tied by ropes or whether animals had access to the forest at will or occasionally with their owners. Blood samples for serology were obtained from these dogs twice; once in the first campaign, and later in the last campaign.

Tick identification

Adult ticks were identified according to the protocol of Barros-Battesti *et al*. (2006), whereas *Amblyomma* nymphs were identified following that of Martins *et al*. (2010). Due to the absence of a proper literature, *Amblyomma* larvae were identified by direct comparisons with laboratory-reared larvae from engorged females of *A. ovale*, *A. fuscum*, *A. naponense* and *A. brasiliense* (the origins of these engorged females have been described elsewhere) (Martins *et al*. 2010). Some larvae were reared to the nymphal stage in the laboratory; then they were identified following the protocol of Martins *et al*. (2010), which confirmed previous larval identifications. Larvae and nymphs of the genus *Ixodes* were identified to species according to the protocol of Marques *et al*. (2004).

Serology

Canine, rodent and marsupial sera were tested by the indirect immunofluorescence assay (IFA) using

https://doi.org/10.1017/S0031182012002065 Published online by Cambridge University Press

crude antigens derived from 5 *Rickettsia* isolates from Brazil (*R. bellii* strain Mogi, *R. amblyommii* strain Ac37, *R. rhipicephali* strain HJ5, *R. rickettsii* strain Taiaçu and *R. parkeri* strain At24), as previously described (Labruna *et al*. 2007). After isolation of *Rickettsia* at the study site (Atlantic rainforest genotype) sera were also tested against such antigens. Briefly, sera were diluted in 2-fold increments with phosphate-buffered saline (PBS), starting from the 1:64 dilution. Slides were incubated with fluorescein isothiocyanate-labelled rabbit anti-dog IgG (Sigma, St Louis, MO, USA), goat anti-mouse IgG (Sigma, St Louis, MO, USA) and sheep anti-opossum IgG (CCZ, São Paulo, Brazil) for canine, rodent and marsupial sera, respectively. For each sample, the endpoint IgG titre reacting with each of the 5 *Rickettsia* antigens was determined. An endpoint titre at least 4-fold higher for a *Rickettsia* species than that observed for any other *Rickettsia* species was considered probably homologous to the first *Rickettsia* species or to a very closely related species (Labruna *et al*. 2007). In each slide, a serum previously shown to be non-reactive (negative control) and a known reactive serum (positive control) were tested at the 1:64 dilution.

Haemolymph test and PCR

Live ticks were checked individually for the presence of *Rickettsia*-like organisms, using the haemolymph test with Giménez staining (Giménez, 1964; Burgdorfer, 1970), and then frozen at −80 °C. A sample of ticks ($n=8$) that was found to be positive for *Rickettsia*-like organisms in the haemolymph test was selected for rickettsial isolation in cell culture (see below). From all other frozen ticks, DNA was extracted using the guanidine isothiocyanate phenol technique (Sangioni *et al*. 2005) and tested for *Rickettsia* by 2 different PCR protocols. Firstly, all DNA samples were tested with primers CS-78 and CS-323 targeting a relatively conserved fragment of the citrate synthase gene (*gltA*) that occurs in all *Rickettsia* species (Labruna *et al*. 2004). Samples yielding a visible amplicon of the expected size (compatible with 401 bp) were then tested by a second PCR protocol with primers Rr190.70F and Rr190.701R, which amplify a 632 bp fragment of the 190 kDa outer membrane protein gene (*ompA*) (Roux *et al*. 1996) from only some *Rickettsia* species belonging to the SFG. The PCR products were sequenced and submitted to BLAST analysis to determine their similarities to the relevant sequences from identified *Rickettsia* species (Altschul *et al*. 1990). A similar procedure was used to test for *Rickettsia* in tissue samples of small mammals; however, for DNA extraction the DNeasy tissue kit (Qiagen, Chatsworth, CA, USA), was used according to the manufacturer's instruction.

Isolation of rickettsiae in cell culture

The shell-vial technique, as previously described by Marrero and Raoult (1989), and modified by Labruna *et al*. (2004), was used to isolate rickettsiae from adult ticks previously found to be positive for *Rickettsia*-like organisms in the haemolymph tests. Briefly, cultures of Vero cells were inoculated with tick-body homogenates and each tick, split into 2 samples, was incubated at both 28 °C and 32 °C. The percentage of Vero cells infected with rickettsiae was monitored by the use of Giménez staining of cells scraped from each inoculated monolayer. After the establishment of each isolate in the laboratory (i.e. at least 3 cell passages, with the prevalence of infected cells exceeding 90%), rickettsial DNA was extracted from the infected cells (Labruna *et al*. 2004). The extracted DNA was tested in a battery of different PCR protocols, using primer pairs targeting fragments of the rickettsial genes *gltA* and *ompA* as stated above, and for a 856-bp fragment of the rickettsial 135 kDa outer membrane protein (*ompB*) (Roux and Raoult, 2000), and a 834 bp fragment of the *gltA* gene (Labruna *et al*. 2004). All PCR products were purified and sequenced in an automatic sequencer (ABI Prism 310 Genetic; Applied Biosystems/Perkin Elmer, Foster City, CA, USA) according to the manufacturer's protocol. Partial sequences obtained were subjected to BLAST analysis, to determine their similarities to the relevant sequences from identified *Rickettsia* species.

Data analysis

Prevalence and mean intensity of tick infestation on main host species were determined according to the method of Bush *et al*. (1997). The association between canine seropositivity to *Rickettsia* and access to forest or with infestation with at least 1 *A. ovale* tick was evaluated by two-sided Chi-square test. The association between dog parasitism with at least 1 *A. ovale* or *R. sanguineus* tick, and access to forest or restriction to a household area was evaluated in the same way. Values were considered significant at $P<0{\cdot}05$.

RESULTS

Ticks

Eight tick species were collected from the vegetation (Table 1). Regarding the animals, 38 dogs were examined repeatedly according to animal availability at each field campaign, totalling 119 canine examinations for the whole study. *Amblyomma ovale* was the main tick species of this host (743 adults, 32 nymphs and 9 larvae were collected), followed by *R. sanguineus* (178 adults and 19 nymphs). Generally, dogs more restricted to household surroundings were the ones

https://doi.org/10.1017/S0031182012002065 Published online by Cambridge University Press

Table 1. Host-questing ticks collected on the vegetation of the Atlantic rainforest Reserve 'Estação Ecológica de Juréia-Itatins' in Peruibe Municipality, state of São Paulo, Brazil, from April 2010 to February 2011

	No. of tick specimens			
Tick species	Larvae	Nymphs	Adults	Total
Amblyomma ovale	—	—	38	38
Amblyomma brasiliense	—	119	20	139
Amblyomma naponense	—	15	9	24
Haemaphysalis juxtakochi	—	4	2	6
Amblyomma nodosum	1*	3	—	4
Amblyomma cajennense	—	2	1	3
Amblyomma longirostre	—	1	1	2
Amblyomma fuscum	—	1	—	1
Amblyomma spp.	62†	—	—	62

* Refers to a larval cluster that was reared to the nymphal stage in the laboratory.
† Through morphological examinations, these larvae were likely to represent *A. brasiliense* and/or *A. naponense*.

that harboured *R. sanguineus* ticks. Mixed infestation prevalence of dogs with the aforementioned tick species encompassed 11·8% of the canine examinations. Other tick species collected from dogs were *A. naponense* (18 nymphs), *A. brasiliense* (27 nymphs and 2 larvae (reared to the nymphal stage)), *A. fuscum* (5 larvae) and *A. cajennense* (2 nymphs). Sixty-one larvae from dogs were retained as *Amblyomma* spp., but they were likely to represent *A. brasiliense* and/or *A. naponense*.

A total of 65 small mammals were captured by trapping during the study. Ticks collected from these small mammals are presented in Table 2. The prevalence and mean intensity of tick infestation on main host species are presented in Table 3. Dogs displayed the highest prevalence and highest mean intensity of infestation with adults of *A. ovale*. On the other hand, the rodent *E. russatus* was the main host for *A. ovale* subadults, particularly nymphs.

Unplanned tick collections during the study yielded *A. ovale* (1 adult) on a domestic cat, *Dermacentor nitens* (26 adults, 1 nymph) and *A. cajennense* (4 adults) on 6 horses, *A. ovale* (1 adult) on a coati (*Nasua nasua*) and *A. fuscum* (3 males) on a lizard (*Tupinambis merianae*). All of these animals had direct contact with the forest, except for the 6 horses, which were restricted to a mixed overgrowth pasture adjacent to the forest.

Serology

Overall 88·6% of the dogs reacted to at least 1 rickettsial antigen. Generally, canine endpoint titres were highest against *R. parkeri*, with 1 animal displaying an endpoint titre as high as 32768. It was found that 7 dogs and 1 dog respectively had endpoint titres to *R. parkeri* and *R. rickettsii*, at least 4-fold higher than those observed for the other *Rickettsia* species, indicating a possible homologous reaction to these *Rickettsia* species or closely related species. Three of these dogs, when sampled again at the end of the study, retained 4-fold higher titres to *R. parkeri* (Table 4). Using the same criterion, 3 *E. russatus* were exposed to *R. parkeri* or a closely related species, 2 opossums and 1 *Akodon* sp. to *R. rickettsii* or a closely related species, and 1 *Monodelphis* sp. was exposed to *R. bellii* or a closely related species. Comparative serology conducted simultaneously with *Rickettsia* sp. Atlantic rainforest genotype (isolate P-51; see below) and *R. parkeri* (strain At24) antigens with sera from animals from this study yielded identical endpoint titres.

Data analysis

Canine seropositivity for *Rickettsia* spp. was significantly associated with access to forest ($P<0·001$) and with infestation with at least 1 *A. ovale* tick ($P<0·010$). Infestation of dogs with *A. ovale* ticks was significantly associated with access to forest ($P<0·001$), whereas no significant ($P=0·777$) association was found between dog infestation with *R. sanguineus* ticks and either access to forest or restriction to household area.

Haemolymph test and isolation of rickettsiae

Rickettsia isolation was attempted in 8 ticks (6 *A. ovale* and 2 *R. sanguineus*) displaying *Rickettsia*-like structures in the haemolymph. *Rickettsia* were successfully isolated and established in Vero cell culture from 4 *A. ovale* ticks only at 28 °C. Rickettsial DNA samples amplified by PCR from these isolates were sequenced and submitted to Blast analyses. Two of the isolates (designated as P-51 and P-240), each from 1 of 2 *A. ovale* ticks were identified as *Rickettsia* sp. strain Atlantic rainforest, since their *gltA* (1078 bp), *ompB* (761 bp) and *ompA* (567 bp) gene fragments were 100% equal to corresponding sequences (GenBank Accession numbers GQ855235, GQ855236, and GQ855237, respectively) of the original strain Atlantic rainforest recently reported to be infecting a human in the same study site as the present study (Spolidorio *et al*. 2010). One isolate (designated as P-10) from a third *A. ovale* tick was identified as *R. bellii* since its *gltA* fragment (1070 bp), was 100% identical to the corresponding sequence of *R. bellii* in GenBank (CP000087). As expected, no *ompA* or *ompB* PCR products were generated from this isolate, since the primer pairs used for these two genes are known not to work for

https://doi.org/10.1017/S0031182012002065 Published online by Cambridge University Press

Table 2. Tick species collected on small mammals from the Atlantic rainforest Reserve 'Estação Ecológica de Juréia-Itatins' in Peruibe Municipality, state of São Paulo, Brazil, from April 2010 to February 2011

Captured small mammals (*N*)	No. of tick specimens collected according to tick species and developmental stage									
	Amblyomma ovale		*Amblyomma fuscum*		*Amblyomma brasiliense*	*Amblyomma cajennense*	*Ixodes loricatus*			*Amblyomma* sp.*
	larvae	nymphs	larvae	nymphs	nymphs	nymphs	larvae	nymphs	adults	larva
Rodents										
Euryoryzomys russatus (20)	21	23	2	—	—	—	—	1	—	—
Akodon sp. (8)	—	—	10	—	—	—	—	—	—	—
Oxymycterus sp. (2)	—	—	3	—	—	—	—	—	—	—
Sciurus aestuans (1)	—	—	—	3	—	—	—	—	—	1
Olygoryzomys sp. (2)	—	—	—	—	—	—	—	—	—	—
Oecomys sp. (1)	—	—	—	—	—	—	—	—	—	—
Marsupials	—	—	—	—	—	—	—	—	—	—
Didelphis aurita (19)	2	—	93	201	3	1	—	—	47	—
Metachirus nudicaudatus (3)	—	—	2	4	—	—	—	—	4	—
Micoureus demererae (3)	—	—	2	2	—	—	8	2	—	—
Monodelphis spp. (6)	—	—	—	—	—	—	7	2	—	—
Total (65)	23	23	112	207	3	1	15	5	51	1

N, number of captured individuals.
* Could not be identified to species level through morphological examination.

Table 3. Prevalence and mean intensity of tick infestation on animals from the Atlantic rainforest Reserve 'Estação Ecológica de Juréia-Itatins' in Peruibe Municipality, state of São Paulo, Brazil, from April 2010 to February 2011

Tick species	Tick prevalence (mean intensity) per tick stage according to host species*			
	Domestic dogs ($N=119$)#	*Didelphis aurita* ($N=19$)	*Euryoryzomys russatus* ($N=20$)	*Akodon* sp. ($N=8$)
Amblyomma ovale				
Male	59·7 (7·5)	—	—	—
Female	51·3 (3·5)	—	—	—
Nymph	10·1 (2·7)	—	30 (3·8)	—
Larvae	3·4 (2·3)	10·5 (1·0)	20 (5·3)	—
Amblyomma fuscum				
Nymph	—	73·7 (14·4)	—	—
Larvae	1·7 (2·5)	68·4 (7·1)	10 (1·0)	75 (1·7)
Amblyomma brasiliense				
Nymph	10·9 (2·1)	10·5 (0·2)	—	—
Larvae	0·8 (2·0)	—	—	—
Amblyomma naponense				
Nymph	13·4 (1·1)	—	—	—
Amblyomma cajennense				
Nymph	1·7 (1·0)	5·3 (1·0)	—	—
Amblyomma spp.				
Larvae	6·7 (8·3)+	—	—	—
Rhipicephalus sanguineus				
Male	19·3 (4·4)	—	—	—
Female	20·2 (3·2)	—	—	—
Nymph	5·9 (2·7)	—	—	—
Ixodes loricatus				
Male	—	26·3 (1·8)	—	—
Female	—	68·4 (2·9)	—	—
Nymph	—	—	5 (1·0)	—
Larvae	—	—	—	—

* Prevalence: number of tick-infested animals / number of examined animals × 100; Mean intensity: mean number of ticks per infested host.
\# *N*, number of canine examinations, since most dogs were re-sampled during the study period.
\+ larvae morphologically compatible with *A. brasiliense* and *A. naponense*.

Table 4. Seroreactivity to five *Rickettsia* species of animals from the Atlantic rainforest Reserve 'Estação Ecológica de Juréia-Itatins' in Peruibe Municipality, state of São Paulo, Brazil, from April 2010 to February 2011

Animals (no. tested)	No. seroreactive animals to each of the *Rickettsia* species (% seroreactivity for each animal species)					No. animals with determined homologous reaction (PAIHR in parentheses)†
	R. parkeri	*R. rickettsii*	*R. amblyommii*	*R. rhipicephali*	*R. bellii*	
Dogs – 1st sample (35)	28 (80·0)	29 (82·9)	26 (74·3)	27 (77·1)	14 (40·0)	7 (*R. parkeri*) 1 (*R. rickettsii*)
Dogs – 2nd sample (14)	12 (85·7)	12 (85·7)	13 (92·9)	13 (92·9)	9 (64·3)	3 (*R. parkeri*)
Didelphis aurita (17)	16 (94·1)	17 (100)	17 (100)	14 (82·3)	14 (82·3)	2 (*R. rickettsii*)
Euryoryzomys russatus (17)	4 (23·5)	5 (29·4)	5 (29·4)	5 (29·4)	3 (17·6)	3 (*R. parkeri*)
Akodon sp. (8)	3 (37·5)	2 (25·0)	3 (37·5)	2 (25·0)	0	1 (*R. rickettsii*)
Metachirus nudicaudatus (2)	2 (100)	2 (100)	2 (100)	2 (100)	1 (50)	—
Monodelphis sp. (3)	1 (33·3)	1 (33·3)	1 (33·3)	1 (33·3)	2 (66·6)	1 (*R. bellii*)
Oxymycterus sp. (2)	0	0	0	0	0	—
Oecomys sp. (1)	0	0	0	0	0	—

† An homologous reaction was determined when an endpoint titre to a *Rickettsia* species was at least 4-fold higher than those observed for the other *Rickettsia* species. In this case, the *Rickettsia* species (or a very closely related species) involved in the highest endpoint titre was considered the possible antigen involved in a homologous reaction (PAIHR).

https://doi.org/10.1017/S0031182012002065 Published online by Cambridge University Press

Table 5. Rickettsial infection in adult ticks collected from hosts and vegetation in the Atlantic rainforest Reserve 'Estação Ecológica de Juréia-Itatins' in Peruibe Municipality, state of São Paulo, Brazil, from April 2010 to February 2011

Tick species	Source (no. hosts tested/no. hosts with infected ticks)	No. ticks tested by PCR	No. infected ticks (%)	*Rickettsia* species (no. ticks)
Amblyomma ovale	Dog (29/18)	232	33 (14·2)	*Rickettsia* sp.* (30), *R. bellii* (4)**
A. ovale	Cat (1/0)	1	0	—
A. ovale	*Nasua nasua* (1/1)	1	0	—
A. ovale	Vegetation	16	1 (6·3)	*Rickettsia* sp.* (1)
Rhipicephalus sanguineus	Dog (10/2)	98	2 (2·0)	*Rickettsia* sp.* (2)
Amblyomma brasiliense+	Dog (3/1)	8	0	—
A. brasiliense	Vegetation	6	0	—
Amblyomma cajennense+	Dog (1/0)	1	0	—
Amblyomma naponense+	Dog (1/0)	1	0	—
A. naponense	Vegetation	4	0	—
Ixodes loricatus	*Didelphis aurita* (5/5)	16	14 (87·5)	*R. bellii* (14)
I. loricatus	*Metachirus nudicaudatus* (1/1)	1	1 (100)	*R. bellii* (1)
Dermacentor nitens	Horse (3/0)	12	0	—

+ PCR of adult ticks that moulted from engorged nymphs collected on dogs.
* Strain Atlantic rainforest.
** One *A. ovale* tick was infected with both *Rickettsia* sp. strain Atlantic rainforest and *R. bellii*.

R. bellii. Surprisingly, the fourth tick specimen from which we isolated rickettsiae was shown to be infected by both strain Atlantic rainforest and *R. bellii*. In this case, the infected cells were proved to be infected by strain Atlantic rainforest through the *ompA* PCR, which generated a product 100% identical to this strain (GQ855237). On the other hand, analysis of the nucleotide sequence from the *gltA* PCR amplification of this tick revealed dual electropherogram peaks at numerous base positions throughout the nucleotide sequence. Further analysis revealed that the superimposed sequences were consistent with a combination of the known *R. bellii* sequence and the known Atlantic rainforest sequence. Then, this isolate DNA was tested by a new PCR protocol using primers (forward: 5′-ATCCTGATTTGCTGAATTTTTT-3′; reverse: 5′-TGCAATACCAGTACTGACG-3′) that were designed to be specific for a 338-bp fragment of the *R. bellii gltA* gene. In this case, the sequence of the PCR product was 100% identical to *R. bellii* (CP000087), confirming that *R. bellii* was co-infecting the Vero cells together with strain Atlantic rainforest. In summary, the rickettsial stocks generated in the present study derived from *A. ovale* ticks, comprising 2 pure strain Atlantic rainforest isolates, 1 pure *R. bellii* isolate, and 1 mixed culture. These isolates and the mixed culture were deposited at the rickettsial collection of the Faculty of Veterinary Medicine of the University of São Paulo, Brazil. Partial sequences (*gltA, ompB, ompA*) from strain Atlantic rainforest, and partial *gltA* sequence of *R. bellii* (designated as strain Peruibe) generated in this study were deposited into GenBank and assigned nucleotide Accession nos. JQ906783-JQ906786, respectively.

Rickettsial DNA in ticks and hosts

The overall prevalence of rickettsial infection in *A. ovale* ticks from dogs was high (14·2%), with 30 ticks (12·9%) being infected by strain Atlantic rainforest, and 4 ticks (1·7%) infected by *R. bellii* (Table 5). One single tick was infected by both strain Atlantic rainforest and *R. bellii,* as stated above. The prevalence of the infection by strain Atlantic rainforest in *A. ovale* ticks from vegetation was much lower (6·3%). Strain Atlantic rainforest was also found in 2% of the *R. sanguineus* ticks collected from 2 dogs. One of these dogs also had a strain Atlantic rainforest-infected *A. ovale* tick, whereas the other one did not, although it was found to be infested by *A. ovale* ticks. The highest rickettsial infection rate (87·5%) was found for *R. bellii* among *I. loricatus* ticks parasitizing *D. aurita*.

Rickettsial DNA was searched for in spleen and lungs from 15 tick-infested rodents (9 *E. russatus*, 4 *Akodon* spp., and 2 *Oxymycterus* spp.), and 3 marsupials (1 *Micoureus demererounae* and 2 *Monodelphis* sp.). No rickettsial DNA was found in the internal organs of these hosts.

DISCUSSION

In the present study the Atlantic rainforest spotted fever rickettsia, a human pathogen in Brazil, was isolated in cell culture for the first time. Previous phylogenetic analysis inferred from the genes *gltA*, *ompA*, *ompB*, *sca4* and *htrA* (17 kDa protein) revealed that the Atlantic rainforest genotype (also referred to as strain Bahia) is closely related to the human pathogens *R. parkeri*, *R. sibirica* and

https://doi.org/10.1017/S0031182012002065 Published online by Cambridge University Press

R. africae (Spolidorio *et al*. 2010; Silva *et al*. 2011). More recent works have suggested that these 3 *Rickettsia* species could just represent geographical variants of a single species (Walker and Ismail, 2008; Goddard, 2009; Pacheco *et al*. 2012). Until taxonomy of such group is clarified, Atlantic rainforest genotype is considered to be a distinct rickettsial genotype, pathogenic for humans.

The present survey detected rather high infection rates of canine *A. ovale* ticks with Atlantic rainforest genotype. A very high seroprevalence rate of dogs to SFG *Rickettsia*, with titres being highest against *R. parkeri* and *Rickettsia* sp. Atlantic rainforest genotype antigens, was also observed. Moreover, infected *A. ovale* ticks were found on most of the dogs that had access to the rainforest. In fact a highly significant correlation was observed between seropositivity of dogs, access to the rainforest and *A. ovale* infestation. It is thus certain that canine seropositivity is a result of infestations by *A. ovale* ticks acquired during incursions into the forest. Such observation is further strengthened by the detection of infected *A. ovale* ticks questing on vegetation of trails inside the forest.

In a previous study conducted in another Atlantic rainforest reserve of the state of São Paulo, Sabatini *et al*. (2010) reported Atlantic rainforest genotype infecting 13·6% of the *A. ovale* ticks collected from dogs, and 8·8% of *A. ovale* ticks collected from vegetation. In the present study, a higher infection rate by the Atlantic rainforest genotype was also found amongst *A. ovale* ticks taken from dogs (12·9%) in comparison to those picked from vegetation (6·3%). These differences might be attributed to *Rickettsia* activation (Hayes and Burgdorfer, 1982), which results in higher ricketsial load in fed ticks and, consequently, in higher chances of detection by PCR. Although *Rickettsia* activation for Atlantic rainforest genotype is for now speculative, the mean annual temperature of the studied area is at least 17 °C below a dog's temperature. Thus it is reasonable to assume that *Rickettsia* replication is enhanced in ticks feeding on dogs. Whatever the case, determining whether the disease agent can be transmitted to humans by ticks questing on the vegetation but after several hours of parasitism or immediately from dog ticks that accidentally detached and may transmit the already re-activated *Rickettsia* is an important feature to be addressed by forthcoming research.

Alternatively, higher infection rates on feeding ticks could be related to horizontal transmission via dogs. Such horizontal transmission could be between co-feeding ticks on a rickettsemic dog, which is the classic route of horizontal transmission of several other *Rickettsia* species (Philip, 1959; Piranda *et al*. 2011). However, horizontal transmission could also occur via copula in ticks, as reported for other *Rickettsia* and tick species (Hayes *et al*. 1980), or between co-feeding ticks on non-rickettsemic hosts (Zemtsova *et al*. 2010); however, the significance of the role of this latter route appears to be much less efficient than it is for some tick-borne viruses (Labuda and Nuttall, 2004).

Horizontal transmission could also be the source of infection of the *R. sanguineus* ticks (2%) that were found to be infected by the Atlantic rainforest genotype in the present study, since these ticks co-infested dogs with *A. ovale* ticks. Interestingly, Sabatini *et al*. (2010) also found a 1·9% infection rate among *R. sanguineus* taken from *A. ovale*-infested dogs. Anyhow, the *R. sanguineus*–strain Atlantic rainforest relationship is a matter of concern since dog parasitism by both tick species is a common feature and *R. sanguineus* is a nidicolous tick that completes its whole life cycle in human dwellings (Guglielmone *et al*. 2006).

Even though rickettsia was not found in the internal organs of the rodent *E. russatus*, this animal had the highest prevalence and mean intensity of *A. ovale* nymphs infestation among the small vertebrates examined so far. Moreover this rodent also displayed a high seroprevalence rate with titres highest against *R. parkeri*. These findings suggest a role of this animal as an amplifier host for the Atlantic rainforest genotype to *A. ovale* ticks, a condition yet to be evaluated experimentally. On the other hand, even though dogs (the main host for the adult stage of *A. ovale* in the study region) are susceptible to rickettsial infection (as demonstrated by a high infection prevalence and very high serological end-point titres to *R. parkeri*), only adult-stage *A. ovale* ticks feed on dogs. Because transovarial transmission rates are likely to be low or absent when the primary rickettsial infection of ticks occurs during adult feeding (Burgdorfer and Brinton, 1975; Piranda *et al*. 2011), the epidemiological influence of dogs as amplifier hosts of the Atlantic rainforest genotype for *A. ovale* ticks is questionable.

Our mixed cell culture of 2 *Rickettsia* species (Atlantic rainforest genotype and *R. bellii*) from a single *A. ovale* specimen is noteworthy. While multiple infection by 2 or more *Rickettsia* species have been only rarely reported in individual ticks, all previous reports were confirmed solely by PCR performed directly on tick DNA extracts. These previous reports include *R. parkeri* and '*Candidatus* R. andeanae' infecting an *A. maculatum* specimen (Varela-Stokes *et al*. 2011), *R. rickettsii* and *R. amblyommii* infecting an *Amblyomma americanum* specimen (Berrada *et al*. 2011), *R. bellii* and *R. rhipicephali* infecting a *Dermacentor variabilis* specimen (Wikswo *et al*. 2008), and a triple infection by *R. bellii*, *Rickettsia montanensis* and *R. rickettsii* in a *D. variabilis* specimen (Carmichael and Fuerst, 2010). Therefore, our finding represents the first mixed culture of 2 *Rickettsia* species from a single naturally infected tick specimen.

https://doi.org/10.1017/S0031182012002065 Published online by Cambridge University Press

In conclusion, the Atlantic rainforest genotype circulates at relatively high levels among *A. ovale* ticks in the focus of human rickettsiosis in Peruibe. This tick species attaches to dogs that enter the forest and are taken to households by the host. An accidental bite by these ticks falling from dogs or eventually from the vegetation may transmit the Atlantic rainforest genotype to human beings and cause a clinical infection. Several aspects of the maintenance and circulation of the pathogen inside the forest shall be highlighted by further field data coupled with experimental data involving host and parasite target species. Further, even though the relationship of the Atlantic rainforest genotype, *A. ovale*, and the Atlantic rainforest biome is clear, it would be interesting to explore a possible relation of *A. ovale* and Atlantic rainforest genotype in other biomes as well. This tick species has a wide range from Mexico to Argentina (Guglielmone *et al*. 2003) including other biomes in Brazil such as the Cerrado (Szabó *et al*. 2007) and Amazon (Labruna *et al*. 2005).

ACKNOWLEDGEMENTS

The authors are indebted to Instituto Florestal from Secretaria do Meio Ambiente of São Paulo State for permits to conduct this study.

FINANCIAL SUPPORT

This research was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP (grant no. 2010/50113-8) and Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (fellowship to M.P.J.S. and Academic Career Research Fellowship to M.B.L.).

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403–410.

Barros-Battesti, D. M. B., Arzua, M. and Bechara, G. H. (2006). *Carrapatos de Importância Medico-Veterinária da Região Neotropical: um Guia Ilustrado para Identificação de Espécies*. Vox/ICTTD-3/Butantan, São Paulo/Brasil.

Berrada, Z. L., Goethert, H. K., Cunningham, J. and Telford, S. R. 3rd (2011). *Rickettsia rickettsii* (Rickettsiales: Rickettsiaceae) in *Amblyomma americanum* (Acari: Ixodidae) from Kansas. *Journal of Medical Entomology* **48**, 461–467. doi: http://dx.doi.org/10.1603/ME10130.

Burgdorfer, W. (1970). The hemolymph test. *American Journal of Tropical Medicine and Hygiene* **19**, 1010–1014.

Burgdorfer, W. and Brinton, L. P. (1975). Mechanisms of transovarial infection of spotted fever rickettsiae in ticks. *Annals of the New York Academy of Sciences* **266**, 61–72.

Bush, A. O., Lafferty, K. D., Lotz, J. M. and Shostaka, A. W. (1997). Parasitology meets ecology on its own terms: Margolis *et al*. revisited. *Journal of Parasitology* **83**, 575–583.

Carmichael, J. R. and Fuerst, P. A. (2010). Molecular detection of *Rickettsia bellii*, *Rickettsia montanensis*, and *Rickettsia rickettsii* in a *Dermacentor variabilis* tick from nature. *Vector Borne and Zoonotic Diseases* **10**, 111–115. doi:10.1089/vbz.2008.0083.

Conti-Díaz, I. A., Moraes-Filho, J., Pacheco, R. C. and Labruna, M. B. (2009). Serological evidence of *Rickettsia parkeri* as the etiological agent of rickettsiosis in Uruguay. *Revista do Instituto de Medicina Tropical de São Paulo* **51**, 337–339. doi: http://dx.doi.org/10.1590/S0036-46652009000600005.

Cragun, W. C., Bartlett, B. L., Ellis, M. W., Hoover, A. Z., Tyring, S. K., Mendoza, N., Vento, T. J., Nicholson, W. I., Eremeeva, M. E., Olano, J. P., Rapini, R. P. and Paddock, C. D. (2010). The expanding spectrum of eschar-associated Rickettsioses in the United States. *Archives of Dermatology* **146**, 641–648. doi:10.1001/archdrmatol.2010.48).

Gimemez, D. F. (1964). Staining rickettsiae in yolk-sac cultures. *Stain Technology* **39**, 135–140.

Goddard, J. (2009). Historical and recent evidence for close relationships among *Rickettsia parkeri*, *R. conorii*, *R. africae*, and *R. sibirica*: implications for rickettsial taxonomy. *Journal of Vector Ecology* **34**, 238–242. doi:10.1111/j.1948-7134.2009.00032.x.

Guglielmone, A. A., Estrada-Peña, A., Mangold, A. J., Barros-Battesti, D. M., Labruna, M. B., Martins, J. R., Venzal, J. M., Arzua, M. and Keirans, J. E. (2003). *Amblyomma aureolatum* (Pallas, 1772) and *Amblyomma ovale* Koch, 1844 (Acari: Ixodidae): hosts, distribution and 16S rDNA sequences. *Veterinary Parasitology* **113**, 273–288.

Guglielmone, A. A., Szabó, M. P. J., Martins, J. R. S. and Estrada-Peña, A. (2006). Diversidade e importância de carrapatos na sanidade animal. In *Carrapatos de Importância Medico-Veterinária da Região Neotropical: um Guia Ilustrado para Identificação de Espécies* (ed. Barros-Battesti, D. M. B., Arzua, M. and Bechara, G. H.), pp. 115–138. Vox/ICTTD-3/Butantan, São Paulo/Brasil.

Hayes, S. F. and Burgdorfer, W. (1982). Reactivation of *Rickettsia rickettsii* in *Dermacentor andersoni* ticks: an ultrastructural analysis. *Infection and Immunity* **37**, 779–785.

Hayes, S. F., Burgdorfer, W. and Aeschlimann, A. (1980). Sexual transmission of spotted fever group rickettsiae by infected male ticks: detection of rickettsiae in immature spermatozoa of *Ixodes ricinus*. *Infection and Immunity* **27**, 638–642.

Labruna, M. B. (2009). Ecology of Rickettsia in South America. *Annals of the New York Academy of Sciences* **1166**, 156–166. doi:10.1111/j.1749-6632.2009.04516.x.

Labruna, M. B., Camargo, L. M. A., Terrassini, F. A., Ferreira, F., Schumaker, T. T. and Camargo, E. P. (2005). Ticks (Acari: Ixodidae) from the state of Rondônia, western Amazon, Brazil. *Systematic and Applied Acarology* **10**, 17–32.

Labruna, M. B., Horta, M. C., Aguiar, D. M., Cavalcante, G. T., Pinter, A., Gennari, S. M. and Camargo, L. M. A. (2007). Prevalence of *Rickettsia* infection in dogs from the urban and rural areas of Monte Negro Municipality, Western Amazon, Brazil. *Vector Borne and Zoonotic Diseases* **7**, 249–256.

Labruna, M. B., Whitworth, T., Bouyer, D. H., McBride, J. L., Camargo, M. A., Camargo, E. P., Popov, V. and Walker, D. H. (2004). *Rickettsia bellii* and *Rickettsia amblyommii* in *Amblyomma* ticks from the state of Rondonia, Western Amazon, Brazil. *Journal of Medical Entomology* **41**, 1073–1081.

Labuda, M. and Nuttall, P. A. (2004). Tick-borne viruses. *Parasitology* **129** (Suppl.), S221–S245.

Marques, S., Barros-Battesti, D. M., Onofrio, V. C., Famadas, K. M., Faccini, J. L. and Keirans, J. E. (2004). Redescription of larva, nymph and adults of *Ixodes* (*I.*) *loricatus* Neumann, 1899 (Acari: Ixodidae) based on light and scanning electron microscopy. *Systematic Parasitology* **59**, 135–146.

Marrero, M. D. and Raoult, D. (1989). Centrifugation-shell vial technique for rapid detection of Mediterranean spotted fever rickettsia in blood culture. *American Journal of Tropical Medicine and Hygiene* **40**, 197–199.

Martins, T. F., Onofrio, V. C., Barros-Battesti, D. M. and Labruna, M. B. (2010). Nymphs of the genus *Amblyomma* (Acari: Ixodidae) of Brazil: descriptions, redescriptions, and identification key. *Ticks and Tick-borne Diseases* **1**, 75–99. doi:10.1016/j.ttbdis.2010.03.0022.

Medeiros, A. P., de Souza, A. P., de Moura, A. B., Lavina, M. S., Belatto, V., Sartor, A. A., Nieri-Bastos, F. A., Richtzenhain, L. J. and Labruna, M. B. (2011). Spotted fever group Rickettsia infecting ticks (Acari: Ixodidae) in the state of Santa Catarina, Brazil. *Memórias do Instituto Oswaldo Cruz* **106**, 926–930. doi:10.1590/S0074-02762011000800005.

Mertins, J. W., Moorhouse, A. S., Alfred, J. T. and Hutcheson, H. J. (2010). *Amblyomma triste* (Acari: Ixodidae): new North American collection records, including the first from the United States. *Journal of Medical Entomology* **47**, 536–542. doi: http://dx.doi.org/10.1603/ME09165.

Nava, S., Elshenawy, Y., Eremeeva, M. A., Mastropaolo, M. and Paddock, C. D. (2008). *Rickettsia parkeri* in Argentina. *Emerging Infectious Diseases* **14**, 1894–1897.

Pacheco, R. C., Arzua, M., Nieri-Bastos, F. A., Moraes-Filho, J., Marcili, A., Richtzenhain, L. J., Barros-Battesti, D. M. and Labruna, M. B. (2012). Rickettsial infection in ticks (Acari: Ixodidae)

collected on birds in southern Brazil. *Journal of Medical Entomology* **49**, 710–716. doi: http://dx.doi.org/10.1603/ME11217.

Pacheco, R. C., Moraes-Filho, J., Marcili, A., Richtzenhain, L. J., Szabó, M. P., Catroxo, M. H., Bouyer, D. H. and Labruna, M. B. (2011). *Rickettsia monteiroi* sp. nov., infecting the tick *Amblyomma incisum* in Brazil. *Applied and Environmental Microbiology* **77**, 5207–5211. doi:10.1128/AEM.05166-11.

Paddock, C. D., Sumner, J. W., Comer, J. A., Zaki, S. R., Goldsmith, C. S., Goddard, J., McLellan, S. L. F., Tamminga, C. L. and Ohl, C. A. (2004). *Rickettsia parkeri:* a newly recognized cause of spotted fever rickettsiosis in the United States. *Clinical and Infectious Diseases* **38**, 812–813.

Parker, R. R., Kohls, G. M., Cox, G. W. and Davis, G. E. (1939). Observations on an infectious agent from *Amblyomma maculatum*. *Public Health Reports* **54**, 1482–1484.

Philip, C. B. (1959). Some epidemiological considerations in Rocky Mountain spotted fever. *Public Health Reports* **74**, 595–600.

Piranda, E. M., Faccini, J. L., Pinter, A., Pacheco, R. C., Cançado, P. H. and Labruna, M. B. (2011). Experimental infection of *Rhipicephalus sanguineus* ticks with the bacterium *Rickettsia rickettsii*, using experimentally infected dogs. *Vector Borne and Zoonotic Diseases* **11**, 29–36. doi:10.1089/vbz.2009.0250.

Romer, Y., Seijo, A., Crudo, F., Nicholson, W. L., Varela-Stokes, A., Lash, R. R. and Paddock, C. (2011). *Rickettsia parkeri* rickettsiosis, Argentina. *Emerging Infectious Diseases* **17**, 1169–1173. doi:10.3201/eid1707.101857.

Roux, V., Fournier, P. E. and Raoult, D. (1996). Differentiation of spotted fever group rickettsiae by sequencing and analysis of restriction fragment length polymorphism of PCR-amplifed DNA of the gene encoding the protein rOmpA. *Journal of Clinical Microbiology* **34**, 2058–2065.

Roux, V. and Raoult, D. (2000). Phylogenetic analysis of members of the genus *Rickettsia* using the gene encoding the outer membrane protein rOmpB (*ompB*). *International Journal of Systematic and Evolutionary Microbiology* **50**, 1449–1455.

Sabatini, G. S., Pinter, A., Nieri-Bastos, F., Marcili, A. and Labruna, M. B. (2010). Survey of ticks (Acari: Ixodidae) and their Rickettsia in an Atlantic rainforest reserve in the State of São Paulo, Brazil. *Journal of Medical Entomology* **47**, 913–916. doi: http://dx.doi.org/10.1603/ME10073.

Sangioni, L. A., Horta, M. C., Vianna, M. C. B., Gennari, S. M., Soares, R. M., Galvão, M. A. M., Schumaker, T. T. S., Ferreira, F., Vidotto, O. and Labruna, M. B. (2005). Rickettsial infection in animals and Brazilian spotted fever endemicity. *Emerging infectious Diseases* **11**, 265–270.

Silva, N., Eremeeva, M. E., Rozental, T., Ribeiro, G. S., Paddock, C. D., Ramos, E. A. G., Favacho, A. R. M., Reis, M. G., Dasch, G. A., de Lemos, E. R. S. and Ko, A. I. (2011). Eschar-associated spotted fever Rickettsiosis, Bahia, Brazil. *Emerging Infectious Diseases* **17**, 275–278. doi:10.3201/eid1702.100859.

Silveira, I., Pacheco, R. P., Szabó, M. P. J., Ramos, H. G. C. and Labruna, M. B. (2007). *Rickettsia parkeri* in Brazil. *Emerging Infectious Diseases* **13**, 1111–1113.

Spolidorio, M. G., Labruna, M. B., Mantovani, E., Brandão, P., Richtzenhain, L. J. and Yoshinari, N. H. (2010). Novel spotted fever group rickettsioses, Brazil. *Emerging Infectious Diseases* **16**, 521–523. doi:10.3201/eid1603.091338.

Szabó, M. P. J., Olegário, M. M. M. and Santos, A. L. Q. (2007). Tick fauna from two locations in the Brazilian savannah. *Experimental and Applied Acarology* **43**, 73–84.

Terassini, F. A., Barbieri, F. S., Albuquerque, S., Szabó, M. P. J., Camargo, L. M. A. and Labruna, M. B. (2010). Comparison of two methods for collecting free-living ticks in the Amazonian forest. *Ticks and Tick-borne Diseases* **1**, 194–196. doi:10.1016/j.ttbdis.2010.08.002.

Varela-Stokes, A., Paddock, C. D. and Engber, B. M. T. (2011). *Rickettsia parkeri* in *Amblyomma maculatum* ticks, North Carolina, USA, 2009–2010. *Emerging Infectious Diseases* **17**, 2350–2353. doi: http://dx.doi.org/10.3201/eid1712.110789.

Venzal, J. M., Portillo, A., Estrada Peña, A., Castro, O., Cabrera, P. A. and Oteo, J. A. (2004). *Rickettsia parkeri* in *Amblyomma triste* from Uruguay. *Emerging Infectious Diseases* **10**, 1493–1495.

Walker, D. H. and Ismail, N. (2008). Emerging and re-emerging rickettsioses: endothelial cell infection and early disease events. *Nature Review Microbiology* **6**, 375–386.

Wikswo, M. E., Hu, R., Dasch, G. A., Krueger, L., Arugay, A., Jones, K., Hess, B., Bennett, S., Kramer, V. and Eremeeva, M. E. (2008). Detection and identification of spotted fever group rickettsiae in *Dermacentor* species from southern California. *Journal of Medical Entomology* **45**, 509–516.

Zemtsova, G., Killmaster, L. F., Mumcuoglu, K. Y. and Levin, M. L. (2010). Co-feeding as a route for transmission of *Rickettsia conorii* israelensis between *Rhipicephalus sanguineus* ticks. *Experimental and Applied Acarology* **52**, 383–392. doi:10.1007/s1049301093757.